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Prokaryotic BirA ligase biotinylates K4, K9, K18 and K23 in histone H3 †

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BirA ligase is a prokaryotic ortholog of holocarboxylase synthetase (HCS) that can biotinylate proteins. This study tested the hypothesis that BirA ligase catalyzes the biotinylation of eukaryotic histones. If so, this would mean that recombinant BirA ligase is a useful surrogate for HCS in studies of histone biotinylation. The biological activity of recombinant BirA ligase was confirmed by enzymatic biotinylation of p67. In particular, it was found that BirA ligase biotinylated both calf thymus histone H1 and human bulk histone extracts. Incubation of recombinant BirA ligase with H3-based synthetic peptides showed that lysines 4, 9, 18, and 23 in histone H3 are the targets for the biotinylation by BirA ligase. Modification of the peptides (e.g., serine phosphorylation) affected the subsequent biotinylation by BirA ligase, suggesting crosstalk between modifications. In conclusion, this study suggests that prokaryotic BirA ligase is a promiscuous enzyme and biotinylates eukaryotic histones. Moreover the biotinylation of histones by BirA ligase is consistent with the proposed role of human HCS in chromatin. [BMB reports 2008; 41(4): 310-315]

INTRODUCTION

Biotin is a coenzyme for five biotin-dependent carboxylases: acetyl-CoA carboxylases 1 and 2, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase (1). Holocarboxylase synthetase (HCS) mediates the binding of biotin to specific lysine (K) residues in carboxylases (1-3). HCS was first purified from bovine liver cytosol and characterized by Chiba *et al*. (4). Moreover, HCS has been detected in the cytoplasm, mitochondria, and nuclei (5-8).

Prokaryotes, such as *Escherichia coli*, express an ortholog of human HCS, BirA ligase, which mediates the binding of biotin to the biotinyl carboxyl carrier protein, a bacterial counterpart of acetyl-CoA carboxylase (9). The biotinylation of apocarboxylases by both HCS and BirA ligase occurs through the addition of a biotin molecule to a specific lysine residue located in a conserved Met-Lys-Met domain (2, 10).

HCS and BirA ligase also share the following features, which theoretically can make BirA a good model for examining protein biotinylation by HCS. BirA ligase plays a key role in cell signaling and chromatin remodeling during biotin biosynthesis in prokaryotes (11, 12). Similar mechanisms of gene regulation for HCS have been reported in eukaryotes. First, biotinyl-AMP appears to regulate the transcription of genes coding propionyl-CoA carboxylase and pyruvate carboxylase in human liver cells through an unknown mechanism of action (13). Second, the biotinylation of histones by HCS and biotinidase (BTD) (6, 14) is associated with gene repression (8). For example, biotin increases the biotinylation of K12 in histone H4 (K12Bio H4) at promoter 1 of the *sodium-dependent multivitamin transporter* gene, which represses transporter transcription (15).

K12 in histone H4 is one of several lysine residues on different histones that can be biotinylated *in vitro* by BTD, and one of several histone-specific biotinylation that occur *in vivo* (7, 16, 17). Despite the significance of these findings, it was suggested that HCS might be more important for biotinylating histones than BTD (6, 18). For example, the knockdown of HCS in *Drosophila melanogaster* decreased the abundance of biotinylated histones, which affected the gene expression patterns and stress resistance in flies. In contrast, the knockdown of BTD did not affect these variables to a significant extent (18, 19). Recombinant, bioactive HCS is difficult to produce because it generally does not fold correctly under standard conditions (data not shown). This study tested the hypothesis that histones are targets for biotinylation by BirA ligase, and that BirA ligase is a useful surrogate for HCS in studies of histone biotinylation *in vitro*.

RESULTS

The identity of recombinant p67 was confirmed by sequencing

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the plasmid, "p67-pET30a", and by molecular weight analysis after gel electrophoresis (Fig. 1A, lane 1). The purified p67 in the eluates from the His-Trap FF columns showed a strong biotinylation signal (Fig. 1A, lane 2). The biotinylation of recombinant p67 was attributed to the activity of endogenous BirA ligase in *E. coli*. In subsequent experiments, p67 was chromatographed over immobilized avidin, which yielded a pure, essentially non-biotinylated fraction of recombinant p67 (Fig. 1A, compare lanes 2 and 4). However, it should be noted that $<10\%$ of p67 in the starting material was biotinylated based on the observation that similar amounts of p67 were detected by Coomassie blue staining before and after the avidin column (Fig. 1A, compare lanes 1 and 3). A time-dependent increase in the biotinylation of p67 was observed when biotin-free p67 was incubated with the recombinant BirA ligase for up to 2 minutes at room temperature (Fig. 1B). These results are consistent with the hypothesis that both recombinant BirA ligase and its substrate p67 are biologically active. Having confirmed the activity and specificity of recombinant Bir A ligase, its ability to biotinylate intact histones and short peptides was examined based on histone amino acid sequences.

The biotinylation of histone H1 from a calf thymus increased in a time-dependent manner when incubated with BirA ligase and cofactors for up to 180 minutes (Fig. 2A). The histone extracts from Jurkat cells (containing histones H1, H2A, H2B, H3 and H4) were biotinylated by BirA ligase in a time-dependent manner (Fig. 2B). An equal loading of the lanes was confirmed by Coomassie blue staining (Fig. 2C). This suggests that prokaryotic BirA ligase can biotinylate all five major classes of human histones.

Finally, recombinant BirA ligase was used as a model to identify the amino acid residues in human histones that would be potential targets for biotinylation by HCS. The first peptides incubated with BirA ligase were denoted $N_{1\text{-}25}$ (spanning amino acids 1-25 in human histone H3) and $N_{15\text{-}39}$ (spanning amino acids 15-39). The biotinylation signals in peptides $N_{1\text{-}25}$ and $N_{15\text{-}39}$ had comparable strength (Fig. 3A, lanes 1 and 2), suggesting that the N-terminus in histone H3 contains the targets for biotinylation by BirA ligase. Next, BirA ligase was incubated with peptide $C_{116\text{-}136}$ (spanning amino acids 116 through 136 in the C-terminus from histone H3). $C_{116\text{-}136}$ contains a lysine residue in position 122. No biotinylation was detected (Fig. 3A, lane 3), which is consistent with previous observations (16). Therefore, $C_{116\text{-}136}$ was subsequently used as a negative control.

The next series of peptides incubated with biotin and BirA ligase focused on shorter amino acid sequences from the N-terminus to identify potential biotinylation sites and to understand the effects of the neighboring amino acid residues on the strength of biotinylation. Short peptides were synthesized based on the sequence of the 25 N-terminal amino acids of human histone H3, as described elsewhere (16). Peptide $N_{1\text{-}9}$ included amino acids 1-9 of the N-terminal tail (ARTKQTARK). The biotinylation of $N_{1\text{-}9}$ was detectable only after 2 h of incubation with BirA ligase (Fig. 3B). However, the strength of the signal at 2 h was high enough to suggest that K4 and K9 might be targets for biotinylation by BirA ligase. Peptide $N_{9\text{-}16}$ included amino acids 9-16 of histone H3 (KSTGGKAP). This peptide is a relatively poor target for biotinylation by BirA ligase (Fig. 3C) compared with peptide $N_{1\text{-}9}$ (Fig. 3B). Peptide $N_{16\text{-}23}$ included amino acids 16-23 of histone H3 (PRKQLATK) and is a good target for biotinylation by BirA ligase. In this

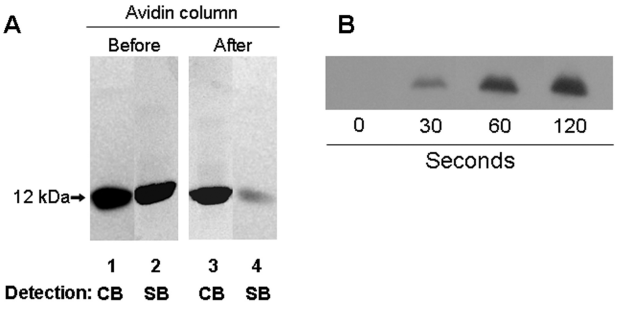

Fig. 1. (A) Recombinant p67 undergoes biotinylation by endogenous BirA ligase. Abbreviations: CB = coomassie blue; SB = streptavidin blotting. (B) Time course for the biotinylation of recombinant p67 by recombinant BirA ligase. The times denote the seconds of incubation of BirA ligase and p67 with biotin and cofactors. Note that all lanes shown here were from the same blot, but that the order of the lanes has been changed electronically to allow comparisons.

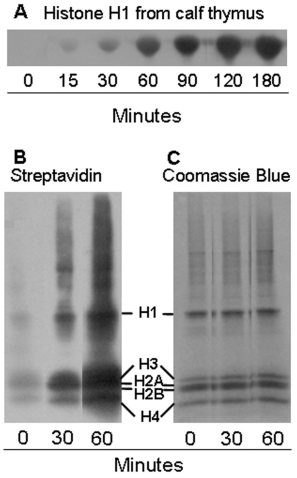

Fig. 2. (A) Time course of biotinylation of histone H1 from calf-thymus by recombinant BirA ligase. (B) Biotinylation of human histones by BirA ligase. The histones were extracted from Jurkat cells and incubated with BirA ligase, biotin, and cofactors for the indicated times. The histones were resolved by gel electrophoresis and the biotin in the transblots was probed with streptavidin. (C) Equal loading of lanes was confirmed by coomassie staining blue. The minutes of incubation of BirA ligase and histones H1 with biotin and cofactors are shown. Note that all lanes shown were from the same blot, but that the order of the lanes was changed electronically to allow comparisons.

case, biotinylation was detectable after 30 minutes incubation with BirA ligase (Fig. 3D), suggesting that K18 and K23 are potential targets for biotinylation by BirA ligase. Peptide N_{18-25} also included K18 and K23 but was found to be a weaker target for biotinylation by BirA ligase (Fig. 3E) than N_{16-23} (Fig. 3D). The differences in biotinylation between peptides N_{16-23} and N_{18-25} can be explained by the effects of the neighboring amino acids, which apparently interferes with Bir A ligase catalyzed biotinyaltion of the peptide-lysine residues.

No biotinylation was detectable when both K4 and K9 in the N-terminal 9 amino acids were replaced with alanine, ($K4,9A_{1-9}$, negative control) (Fig. 4A, lane 1). Peptides containing only K9 or K4 were biotinylated efficiently by Bir A ligase (Fig. 4A, lane 2 and 3), indicating that both lysines are good targets for biotinylation.

Next, this study focused on amino acids 9-16 in the N-terminus of H3. No biotinylation signal was detected when both K9 and K14 in the N-terminal amino acids were replaced with alanine ($K9,14A_{9-16}$) (Fig. 4A, lane 4). However, a strong biotinylation signal was detected when only K14 was replaced with alanine ($K14A_{9-16}$),(Fig. 4A, lane 5), suggesting that K9 is a good target for biotinylation by BirA ligase. Only a weak biotinylation signal was detected when only K9 was replaced with alanine ($K9A_{9-16}$) (Fig. 4A, compare lanes 5 and 6), which suggests that K14 is a moderate target for biotinylation.

Similar experiments on K18 and K23 of histone H3 showed no detectable biotinylation signal when both K18 and K23 were replaced with alanine (K18, $23A_{18-23}$) (Fig. 4A, lane 7). However, a weak biotinylation signal was detected when only K23 was replaced with alanine ($K23A_{23}$) (Fig. 4A, lane 8), indicating that K18 is a moderate target for biotinylation by BirA ligase. A strong biotinylation signal was detected when only K18 was replaced with alanine ($K18A_{16-23}$) (Fig. 4A, lane 9), suggesting that K23 is also a good target for biotinylation by BirA ligase. Likewise, no biotinylation signal was detected when both K18 and K23 were replaced with alanine in peptide $K18,23A_{18-25}$ (Fig. 4A, lane 10). A weak biotinylation signal was detected when K23 was replaced with alanine ($K23A_{18-25}$) (Fig. 4A, compare lanes 11 and 8). A weak biotinylation signal was detected when K18 was replaced with alanine ($K18A_{18-25}$) (Fig. 4A, lane 12). These results were different from to the biotinylation of peptide $K18A_{16-23}$, which suggests that amino acids 16 and 17, and 24 and 25 might affect the biotinylation of K18 and K23 by BirA ligase.

Previous studies suggested that some modification of amino acids can affect the biotinylation of the adjacent lysines. For example, the phosphorylation of S10 in histone H3 caused a decrease in the biotinylation of K9 (16). This study examined whether or not modifications of amino acids affect the biotinylation of the neighboring lysine residues by BirA ligase. For proof of principle, this study focused on the modifications and sequence variations in amino acids surrounding the biotinylation target K9.

A peptide spanning amino acids 6-13 in the N-terminus (N_{6-13}) was found to be a good target for biotinylation (Fig. 4B, lane 1). If K9 was replaced with alanine ($K9A_{6-18}$), the bio-

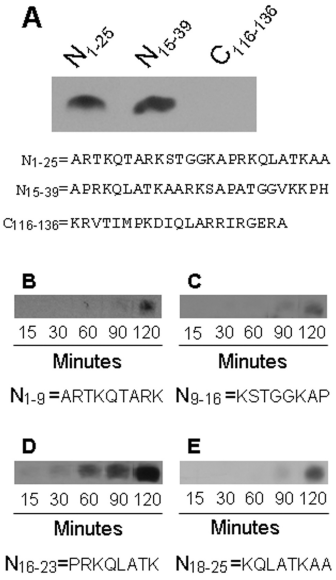

Fig. 3. (A) The N-terminal tail in human histone H3 is the primary target for biotinylation by BirA ligase rather than the C-terminus. Synthetic peptides spanning amino acids 1-25 (lane 1), 15-39 (lane 2), and 116-136 (lane 3) in histone H3 were incubated with BirA ligase, biotin, and cofactors for 1 h. The peptides were resolved by gel electrophoresis and the peptide-bound biotin was probed using streptavidin peroxidase. (B-E) Biotinylation targets of BirA ligase in the N-terminal tail of human histone H3. Note that all lanes shown were from the same blot, but that the order of the lanes was changed electronically to allow comparisons.

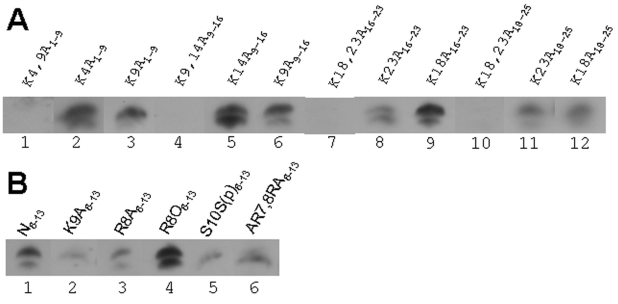

Fig. 4. (A) Biotinylation of K4, K9, K18, and K23 in the N-terminal tail in histone H3. Lanes 1-12 show the synthetic peptides based on the overlapping sequences from the first 25 amino acids of histone H3. These peptides were incubated with BirA ligase, biotin, and cofactors. The peptides were resolved by gel electrophoresis and probed with streptavidin peroxidase. (B) Amino acid modifications and substitutions affect biotinylation of adjacent K9 in histone H3 by BirA ligase. The peptides based on amino acids 6-13, which includes K9 in histone H3, were incubated with BirA ligase, biotin, and cofactors for 2 h. Note that all lanes shown were from the same blot, but that the order of the lanes was changed electronically to allow comparisons.

tinylation signal was at approximately the level of background noise (Fig. 4B, lane 2; negative control). This is consistent with the observations described above in that K9 is a target for biotinylation. Previous studies suggested that R8 interferes with the biotinylation of K9 by BTD (16). In contrast, R-to-A substitution in position 8 ($R8A_{6\text{-}13}$) only improved the biotinylation of K9 by BirA ligase slightly (Fig. 4B, compare lanes 2 and 3). Substitution of ornithine for R8 maintained the positive charge at position 8 ($R8O_{6\text{-}13}$), which resulted in a strong biotinylation signal (Fig. 4B, lane 4). The observations that charge interactions affect histone biotinylation by BirA ligase was further corroborated by the following finding. The level of biotinylation decreased to the background levels when a negative charge was introduced to position 10 by serine phosphorylation ($S10S(p)_{6\text{-}13}$) (Fig. 4B, lane 5). Likewise, there was a decrease in the level of biotinylation when positively charged R8 was moved away from K9 biotinylation compared with the native sequence (Fig. 4B, compare lanes 1 and 6).

DISCUSSION

The covalent binding of biotin to histones in humans is believed to be mediated by BTD and HCS (6, 14). BTD was previously used to identify K4, K9, and K18 as targets for biotinylation in human histone H3. More recently, it was suggested that HCS is more important for the biotinylation of histones (6, 18). Consequently, our previous studies of enzymatic biotinylation of histones were reassessed using recombinant BirA ligase as a surrogate for human HCS. In these studies, a series of novel observations was made. It was demonstrated that the polypeptide p67, which is frequently used to confirm biological activity of BirA ligase and HCS, is biotinylated by endogenous BirA ligase in *E. coli*. Therefore, it is important to purify p67 by avidin chromatography before using it in biotinylation assays. Second, BirA ligase is an non-selective enzyme that is capable of biotinylating human histones, lending further support to the hypothesis that HCS plays an important role in histone biotinylation. Studies pursuing the generation of recombinant bioactive HCS to provide additional support for roles of HCS in chromatin biology are currently underway. Third, K23 was found to be a novel biotinylation site by BirA ligase, in addition to confirming K4, K9, and K18 as targets for biotinylation (16). Fourth, the biotinylation of lysine residues is subject to crosstalk between neighboring amino acids. For example, the biotinylation of K9 is prevented by the phosphorylation of S10, which is a modification known to play a role in mitotic and meiotic chromosome condensation (20). Another example of crosstalk is not through histone modifications per se but by charge patches in chromatin. For example, the removal of positive charges or the introduction of negative charges in the vicinity of K9 is associated with a decrease in the biotinylation of K9.

There is evidence suggesting that streptavidin might bind to non-biotininylated histones (21). This is not a concern in the data presented here because no meaningful signal was detected when the histones were probed with streptavidin prior to incubation with BirA ligase, (Fig. 2B). This is consistent with previous studies of biotin-depleted histones (16, 17).

The possibility that HCS behaves in a different manner to BirA ligase with regard to the biotinylation of histones cannot be excluded. However, three of the biotinylation targets reported in this study (K4, K9, and K18) have already been detected in eukaryotic cells using site-specific antibodies (16). It was further demonstrated that the knockdown of HCS in Drosophila and human lymphoid cells is associated with a substantial decrease in histone biotinylation (15, 18). Importantly, biotinylated histone H3 is the most abundant class of biotinylated histones in Drosophila, and the knockdown of HCS in flies is associated with phenotypes such as a decreased life span and stress resistance (19).

MATERIALS AND METHODS

BirA ligase

Generating recombinant bioactive human HCS is difficult. Therefore, this study used recombinant BirA ligase from *E. coli* as a surrogate for HCS to identify the amino acid residues in histone H3 that are targets for biotinylation. Recombinant, purified BirA ligase was a generous gift from John E. Cronan Jr., University of Illinois at Urbana-Champaign (22).

Recombinant p67

The polypeptide, p67, which comprising the 67 C-terminal amino acids from human propionyl-CoA carboxylase(GenBank accession #AAA60035) including the biotin-binding site (K669) (23), was cloned from Jurkat cell cDNA using the forward primer, 5′-GAATTCCTGCGTTCCCCGATG-3′, and the reverse primer, 5′-GGATCCTCATTCCAGCTCCACGAGCAG-3′, and the vector pSTBlue (Novagen; San Diego, CA). The resulting plasmid was named "p67-pSTBlue." p67-pSTBlue was sequence verified at the University of Nebraska-Lincoln, DNA core facility. The insert encoding p67 was subcloned into vector pET30a (Novagen; San Diego, CA) using EcoR1 and BamH1. This plasmid was named "p67-pET30a." When IPTG was added at a final concentration of 1 mM, the Rosetta 2 (DE3) cells were transformed with p67-pET30a and grown at 37°C to an optical density of 0.3 to 0.6 at 600 nm. Incubation was continued for 16 h. Cell pellets were produced by centrifugation the cell medium at 2,700 g for 30 min. The pellets were resuspended in 10 ml of 50 mM Tris buffer, pH 7.5 and sonicating three times on ice at maximum power at 30-sec intervals (Aquasonic 250 T; VWR). The samples were centrifuged at 2,700 g for 30 min to remove the cell debris. The supernatant was collected and the protein was purified using His Trap FF columns on a AKTA protein purification system according to the manufacturer's protocol (Amersham; Piscataway, NJ). Aliquots of the chromatographic fractions were separated on 18% polyacrylamide gels, and stained with Coomassie blue to

identify the fractions containing p67. Subsequent studies showed that recombinant p67 contained covalently bound biotin (see Results), as judged by gel electrophoresis and streptavidin blotting (24). The biotinylated fraction of p67 was removed using avidin beads. Briefly, 10 ml of recombinant p67 from the His Trap FF column eluate was chromatographed over 3 ml of immobilized avidin (Pierce; Rockford, IL). The non-biotinylated p67 in the column flow-through was collected and quantified by measuring the absorbance at 280 nm.

Biotinylation of p67, histones, and histone-based peptides by BirA ligase

Previously, a procedure for identifying the amino acids that are targets for biotinylation in histones was developed using synthetic peptides and human serum as a source of BTD (17). Both amino acid substitutions and the incorporation of modified amino acids (e.g., phosphorylation of serine) in the peptide chain were used to determine their effects on the biotinylation of the adjacent lysine residues (16, 17). Here, an analogous procedure was used to identify the lysine residues in the histones that are targets for biotinylation by BirA ligase.

The following substrates were tested as targets for biotinylation by BirA ligase: (i) biotin-free p67 was prepared as described above; (ii) bulk extracts of histones were prepared from human lymphoid (Jurkat) cell nuclei using 1 M HCl (24); (iii) commercial calf-thymus histone H1 was purchased from Calbiochem (La Jolla, CA); and (iv) synthetic peptides were produced based on the amino acid sequence in human histone H3 and quantified as described elsewhere (16). The biotinylation assays were carried out as follows: 0.75 μg of p67, 50 μg of commercial histone H1, 0.5 μg or 0.8 μg of synthetic peptides (see below) were incubated with 350 μg of BirA ligase in 100 μl of buffer containing 50 mM Tris acetate (pH 7.5), 0.2 mM biotin, 0.2 mM DTT, 5 mM ATP, and 100 mM $MgCl_2$. The samples were incubated at room temperature or 37°C for up to 180 minutes, depending on the substrate. The reaction was quenched by adding Tris-Glycine or Tricine loading buffer (Invitrogen) and heating the mixture to 95°C for 10 min. After enzymatic biotinylation, the histones were resolved using 18% Tris-Glycine. p67 and the peptides were then resolved using 16% Tricine gels (17). The histones, p67, and proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with streptavidin-peroxidase (16, 17, 24).

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